J Infect Chemother xxx (2018) 1–6

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Original Article

Doxycycline suppresses *Chlamydia pneumoniae* induced interferon-gamma responses in peripheral blood mononuclear cells in children with allergic asthma

Tamar A. Smith-Norowitz [a, *], Diana Weaver [a], Yitzchok M. Norowitz [a], Margaret R. Hammerschlag [a], Rauno Joks [b], Helen G. Durkin [c], Stephan Kohlhoff [a]

[a] Department of Pediatrics, Division of Infectious Diseases, State University of New York Downstate Medical Center, Brooklyn, NY, 11203, USA
[b] Department of Medicine State University of New York Downstate Medical Center, Brooklyn, NY, 11203, USA
[c] Department of Pathology State University of New York Downstate Medical Center, Brooklyn, NY, 11203, USA

ARTICLE INFO

Article history:
Received 15 December 2017
Received in revised form
30 January 2018
Accepted 7 February 2018
Available online xxx

Keywords:
C. pneumoniae
IFN-γ
IL-4
Asthma

ABSTRACT

Persistent respiratory infections caused by *Chlamydia pneumoniae* have been implicated in the pathogenesis of chronic diseases (e.g. asthma). Antibiotics are used to treat *C. pneumoniae* respiratory infections; however, the use of antibiotics as anti-inflammatory agents in treatment of asthma remains controversial. The current study investigated whether ciprofloxacin, azithromycin, or doxycycline can suppress *C. pneumoniae*-induced production of immunoglobulin (Ig) E or cytokines in peripheral blood mononuclear cells (PBMC) obtained from asthmatic children. Apart from blood, nasopharyngeal swab specimens were also collected to test for the presence of *C. pneumoniae* and/or *M. pneumoniae* (qPCR). PBMC (1.5×10^6) from asthmatic pediatric patients (N = 18) were infected or mock infected for 1 h ± *C. pneumoniae* AR-39 at a multiplicity of infection (MOI) = 0.1, and cultured ± ciprofloxacin, azithromycin, or doxycycline (0.1 or 1.0 μg/mLmL) for either 48 h (cytokines) or 10 days (IgE). Interleukin (IL)-4, interferon (IFN)-γ and IgE levels in supernatants were measured (ELISA). When PBMC were infected with *C. pneumoniae*, IL-4 and IFNγ production increased ($p = 0.06$ and 0.03, respectively); IgE levels were low. The now-elevated levels of IL-4 didn't decrease significantly after addition of ciprofloxacin, azithromycin, or doxycycline. However, infected PBMC IFNγ formation decreased significantly when 0.1 μg/mL doxycycline was employed ($p = 0.04$); no dose of ciprofloxacin or azithromycin had any impact. This inhibitory outcome with doxycycline lends support to the use of tetracyclines as immune modulators and anti-inflammatory medications in treatment of *C. pneumoniae*-infected asthma patients.

1. Introduction

Chlamydia pneumoniae is an obligate intracellular organism [1] that causes respiratory infections in adults and children and has been implicated in the pathogenesis of chronic disease (e.g. asthma) [2]. Prior studies have shown that *C. pneumoniae* can cause prolonged respiratory infection in asthmatic and non-asthmatic subjects [3–5]. Infection with *C. pneumoniae* can activate immune cells (e.g. macrophages, endothelial and epithelial cells) to produce cytokines that may contribute to the pathology observed in asthma [2]. In addition, *C. pneumoniae* infection triggers the production of pathogen-specific IgE in children with chronic respiratory disease, which may lead to inflammation [6].

Declarations and Abbreviations: Ig, immunoglobulin; IFN-γ, Interferon gamma; IL, Interleukin; MIC, minimum inhibitory concentration; CDC, centers for disease control; TNF, tumor necrosis factor; MIP, macrophage inflammatory protein; NFκβ, nuclear factor κβ; LPS, lipopolysaccharide; LTA, lipoteichoic acid.

* Corresponding author. SUNY Downstate Medical Ctr. Dept of Pediatrics, Box 49, 450 Clarkson Ave, Brooklyn, NY, 11203, USA.
E-mail addresses: tamar.smith-norowitz@downstate.edu (T.A. Smith-Norowitz), Diana.weaver@downstate.edu (D. Weaver), yitzchok.norowitz@downstate.edu (Y.M. Norowitz), Margaret.hammerschlag@downstate.edu (M.R. Hammerschlag), rauno.joks@downstate.edu (R. Joks), Helen.durkin@downstate.edu (H.G. Durkin), Stephan.kohlhoff@downstate.edu (S. Kohlhoff).

https://doi.org/10.1016/j.jiac.2018.02.004
1341-321X/

Treatment of atypical bacteria, specifically *C. pneumoniae* and *Mycoplasma pneumoniae,* with antibiotics (macrolides, tetracyclines, quinolones) may have beneficial effects in patients with asthma because of the eradication of acute or persistent *C. pneumonaie* infection [2,7], and potential pleiotropic anti-inflammatory properties in addition to anti-chlamydial activity.

Previous studies in our laboratory investigated the steroid-sparing effect of the tetracycline, minocycline, when given to adults with moderate persistent or severe persistent asthma, in addition to steroid treatment [8]. Minocycline treatment was associated with improvement in pulmonary function and asthma symptoms [8]; the effect was independent of the presence of *C. pneumoniae* infection (culture and serology) [8]. The observed effect may have been attributed to inflammation suppression or eradication of the *C. pneumoniae* [3,9]. We have also previously demonstrated that doxycycline can suppress *C. pneumoniae* mediated increases in ongoing IgE and IL-4 responses by PBMC of patients with asthma [10]; the anti-inflammatory effects of doxycycline observed in that model, were highest in the presence of memory lymphocyte responses to *C. pneumoniae* [10]. It has been speculated that targeting inflammatory mediators (e.g. IgE production) may help improve asthma symptoms [10].

Quinolones (levofloxacin and moxifloxacin), azithromycin and doxycycline are antibiotics which are frequently used for the treatment of *C. pneumoniae* respiratory infections [7]. Ciprofloxacin is significantly less active than moxifloxacin and levofloxacin and is not used to treat *C. pneumoniae* respiratory infections in patients [7]; however, ciprofloxacin has been used in the *in vitro* setting to test drug class, as well as anti-inflammatory activity independent of antimicrobial activity [11].

The role of macrolide antibiotics in asthma is ongoing and the major areas of disagreement include the specific impact of these antibiotics. The present study describes whether the role of macrolide antibiotics is anti-inflammatory (having a non-specific effect) or bactericidal (leading to clearance of pathogens and resolution of asthma symptoms). This was achieved by studying the effect of ciprofloxacin, azithromycin, or doxycycline on *C. pneumoniae* induced cytokine responses (interferon [IFN]-γ and IL-4) in children with allergic asthma, as well as the ability of these drugs to regulate *in vitro* IgE responses.

2. Materials and methods

2.1. Study design

We recruited pediatric patients with allergic asthma (male/female, 8–20 years old) from the outpatient department at SUNY Downstate Medical Center (Brooklyn, NY). Inclusion criteria included a physician's diagnosis of stable asthma without current respiratory infection, or current clinically defined persistent asthma symptoms [12], or both, with elevated serum IgE levels (>100 IU/mL). Asthma diagnosis was made at least one year before study enrollment. Exclusion criteria included a history of chronic immunosuppressive or autoimmune disease, human immunodeficiency virus infection, cancer, recent use of systemic corticosteroids (<30 days), antibiotic use, or immunotherapy, tobacco use within the past year, and incomplete follow-up. All subjects had a nasopharyngeal (NP) swab tested for *C. pneumoniae* and *M. pneumoniae* by PCR. At time of enrollment, all subjects had NP swabs and blood collected, and their clinical data was reviewed.

The SUNY Downstate Medical Center Institutional Review Board (Brooklyn, NY) approved all studies and activities. Informed consent (and assent) was obtained from participants for experimentation with human subjects for use of their blood samples. Procedures were followed in accordance with institutional guidelines involving human subjects. The work described has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

2.2. Immunoglobulin determination: total serum IgE

Peripheral blood (10 mL) was collected at the time of enrollment at the clinic site. Total serum IgE levels were determined in serum using the UniCap Total IgE fluoroenzyme immunoassay (Pharmacia and Upjohn Diagnostics, Freiburg, Germany) performed according to the manufacturer's recommendations (reference range for healthy subject: 20–100 IU/mL). All tests were performed in the Clinical Diagnostic Laboratory at SUNY Downstate Medical Center (Brooklyn, NY).

2.3. Detection of C. pneumoniae-specific IgG antibodies

C. pneumoniae-specific IgG antibodies were measured using the microflourescence (MIF) test (AniLabsystems; Vantaa, Finland), as previously described [13].

2.4. Preparation of C. pneumoniae

C. pneumoniae AR-39 (ATCC 53592; Manassas, VA) was propagated as previously described[15]. Briefly, HEp-2 cell (ATCC CCL-23) monolayers were inoculated with *C. pneumoniae* and grown to high titers by serial passage in HEp-2 cells. *C. pneumoniae* elementary bodies (EB) were purified by Urografin (Schering, Berlin, Germany) density gradient centrifugation and were resuspended in sucrose phosphate glutamate buffer (comprising 74.62 g/l sucrose (Sigma, St Louis, MO), 0.517 g/l KH_2PO_4 (Sigma), 1.643 g/l K_2HPO_4 (Sigma), and 0.907 g/l potassium glutamate (Sigma)). Titers were determined by infecting HEp-2 cells with serial dilutions of EB suspension aliquots, fixing cells at 72 h post infection (p.i.), staining with fluorescein-conjugated murine monoclonal genus-specific anti-lipopolysaccharide monoclonal antibody (Pathfinder, Bio-Rad, Hercules, CA), and counting inclusions per well. Aliquots were frozen at −80 °C until use.

2.5. Cell cultures

PBMC were separated from blood on a Ficoll-Paque (GE Healthcare, Sweden) gradient (density 1.077). The PBMC were carefully removed using a transfer pipette (VWR Scientific, San Francisco, CA). Cells were washed twice in RPMI -1640 medium (Life Technologies/GIBCO, Grand Island, NY) with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA), and resuspended in complete RPMI 1640 (c-RPMI). c-RPMI contained RPMI −1640 Medium HEPES Modification (Sigma) supplemented with 5 mM L-glutamine (Sigma) and 10% FBS (Atlanta Biologicals). Cells were counted on a hemocytometer (Fisher Scientific, Springfield, NJ), and cell viability was evaluated, as judged by trypan blue (Fisher Scientific) exclusion. For each experimental condition PBMC (1.5×10^6/mL) were cultured in duplicate in a 24-well flat bottom plate (1mL/well) (CORNING; Corning, NY) at 37 °C in cRPMI medium in a humidified 5% CO_2 atmosphere for up to 12 days. Cell viability was determined at 0, 48 and 240 h (>98%, 95%, and 90%, respectively), in the absence of any infection with *C. pneumoniae*.

2.6. In vitro infection with C. pneumoniae and treatment with antibiotics

Following a 2hr incubation to allow adherence, PBMC cultures were infected with *C. pneumoniae* (by adding purified EB for 1 h), or

Please cite this article in press as: Smith-Norowitz TA, et al., Doxycycline suppresses *Chlamydia pneumoniae* induced interferon-gamma responses in peripheral blood mononuclear cells in children with allergic asthma, J Infect Chemother (2018), https://doi.org/10.1016/j.jiac.2018.02.004

mock-infected (MI) and/or stimulated in the presence or absence of ciprofloxacin (0.1 or 1.0 μg/mL) (Sigma), azithromycin (0.1 or 1.0 μg/mL) (Sigma) or doxycycline (0.1 or 1.0 μg/mL) (Sigma) for up to 12 days at 37 °C in cRPMI in a humidified 5% CO_2 atmosphere. All antibiotics were serially diluted (1:1, 1:2, 1:4, 1:10) [10] to determine optimal dose and kinetics [10], for suppression (for the purpose of cytokine production. Cytokine assays (IL-4 and IFN-γ) were run using supernatants collected from above cultures. The multiplicity of infection (MOI; 0.1) and time points (48 h p.i. for cytokines [10] and 10 d p.i. for IgE [10]) used for analysis were selected by kinetic and dose response studies (using MOI of 0.01–10) for optimization of the assay, which revealed peak concentrations and clear distinctive profiles for the respective outcome variables at these time points. Adherent cells were stained with a fluorescein-conjugated murine monoclonal genus-specific anti-lipopolysaccharide antibody (Becton-Dickinson (BD) Biosciences, San Jose, CA) to confirm and quantify infection with *C. pneumoniae* at 72 h p.i. Two types of controls were used in infection experiments: identical volumes of heat-inactivated purified *C. pneumoniae* [10] and identical volumes of HEp-2 cell cultures not containing any bacteria processed the same way as the purified *C. pneumoniae* [14] based on dose-response experiments.

2.7. Cytokine (IL-4, IFN-γ) or IgE determination: ELISA

For the *in vitro* quantitative determination of human cytokine or IgE content in cell culture supernatants, solid-phase sandwich ELISA assays were performed using either cytokine (IL-4: IL-4 Human ELISA kit, Thermo Fisher Scientific, Waltham, MA) (IFN-γ: Abcam, Cambridge, MA) or IgE ELISA test kits (Bio Quant, San Diego, CA), according to the manufacturer's recommended procedure.

Cell culture supernatants were collected at either 48 h p.i. (cytokines) [10] or 10 days p.i. (IgE) [10], by centrifugation, and samples were stored at −80° until analysis. All specimens were analyzed in duplicate and standard curves determined. The IgE ELISA was modified for *in vitro* use by using a low-range standard curve; the sensitivity of the assay was determined by using 2 standard deviations above the mean of 10 negative control measurements (0.3 ng/mL). Plates were read using an automated microplate reader (Model ELx800; Bio-Tek Instruments, Winooski, VT), with a 450-nm measurement filter. Optical densities were converted to either IU/mL, ng/mL, or pg/mL (1 IU IgE = 2.4 ng/IgE protein). Detection limits for cytokine assays were: IL-4: <2.0 pg/mL; IFN-gamma: <5.0 pg/mL.

2.8. Quantitative real-time polymerase chain reaction (qPCR) of bacteria in swabs and cultures

Extractions of bacterial DNA from NP swab specimens [15] and PBMC were performed using a QIAAmp DNA Mini-Kit (Qiagen Inc., Valencia, CA), according to manufacturer's recommendation. For PBMC cultures, supernatants (with adherent and non-adherent cells) were collected and bacterial DNA extracted. NP samples were stored in the freezer (−20 °C), until analysis. DNA was extracted (QIAAmp DNA mini kit; Qiagen Inc), as previously described [15,16]. In both cases, specimens were tested for the presence and quantification of *C. pneumoniae* and *Mycoplasma pneumoniae* DNA according to Apfalter et al. [15] and Waring et al. [16], respectively, using TAQMan technology-based qPCR on a Light Cycler 2.0 platform (software version 4.0, Roche Diagnostics Corp, Indianapolis, IN). We considered a specimen from either nostril positive for any bacterial species to represent a positive result.

2.9. Statistical analysis

Data are expressed as means ± SD unless elsewise indicated. A Students *t*-test and a non-parametric Wilcoxon Signed Ranks Test were used to compare differences in means of normally and non-normally distributed data, respectively. The Pearson Correlation Test was used to assess the degree of correlation for continuous variables. A 2-sided p-value < 0.05 was taken to indicate statistical significance in all comparisons. All statistical analyses were performed using Windows v.12.0 software (SPSS Inc., Chicago, IL).

3. Results

3.1. Study population demographics

A total of 18 pediatric asthmatic patients (9 male, 9 female; mean age = 13 [± 4] years [range 8–20 years]) were enrolled in the study. All were classified as having moderate persistent asthma and all had been treated with inhaled corticosteroids. Inhaled corticosteroids included Budesonide 500 mcg or Fluticasone HFA (176, 220 or 440 mcg). Leukotriene modifiers included Monteleukast (5 or 10 mg). Total serum IgE levels were high in the patient population (665 [± 159] IU/mL). Table 1 shows the patient baseline clinical and demographic data.

3.2. Serological and nucleic acid amplification testing for C. pneumoniae

Asthmatic patients had *C. pneumoniae*-specific IgG MIF titers >1:16 with a median titer of 1:32. All patients tested negative for *C. pneumoniae* and *M. pneumoniae*, as determined by qPCR (nasopharyngeal swabs).

3.3. Ciprofloxacin, azithromycin, or doxycycline effect on C. pneumoniae-induced IgE

When PBMC from asthmatic patients were infected with *C. pneumoniae* IgE levels were low (0.3 ng/mL) on day 10 and remained at baseline levels. When *C. pneumoniae*-infected PBMC were cultured with either ciprofloxacin (0.1, 1.0 μg/mL), azithromycin (0.1, 1.0 μg/mL) or doxycycline (0.1, 1.0 μg/mL), IgE levels remained low (similar to baseline on day 10) (Data not shown).

Table 1
Participant characteristics.

Asthma (N = 18)	
Age, mean y	13
Female (%)	50
Male (%)	50
Asthma severity	
Moderate persistent (%)	100
Asthma therapy	
Inhaled corticosteroids (%)[a]	100
Leukotriene modifiers (%)[b]	72
FVC (% predicted)	86
FEV_1 (% predicted)	82
FEV_1/FVC	84
FEF_{25-75} (% predicted)	75
History of atopic dermatitis (%)	32
History of allergic rhinitis (%)	89
Total serum IgE (IU/mL)	665 ± 159

Abbreviation: FVC: mean forced vital capacity; FEV_1: mean forced expiratory volume in 1 s; FEF_{25-75}: mid-flow rate or forced expiratory flow occurring in the middle 50% of the patient's exhaled volume.

[a] Inhaled corticosteroids included Budesonide 500 mcg or Fluticasone HFA (176, 220 or 440 mcg).

[b] Leukotriene modifiers included Monteleukast (5 or 10 mg).

Please cite this article in press as: Smith-Norowitz TA, et al., Doxycycline suppresses *Chlamydia pneumoniae* induced interferon-gamma responses in peripheral blood mononuclear cells in children with allergic asthma, J Infect Chemother (2018), https://doi.org/10.1016/j.jiac.2018.02.004

Thus, *C. pneumoniae* infection/antibiotic treatment had no effect on IgE production *in vitro*.

3.4. Ciprofloxacin, azithromycin or doxycycline effect on *C. pneumoniae*-induced IL-4

When the PBMC from asthmatic patients were infected with *C. pneumoniae*, levels of IL-4 produced were increased (5-fold) from baseline ($p = 0.06$) (Fig. 1). Levels of IL-4 decreased in non-significant manners (i.e., slightly) from these organism-infected levels after addition of ciprofloxacin (1.0 μg/mL), azithromycin (0.1 μg/mL), or doxycycline (0.1 or 1.0 μg/mL). IL-4 levels did not significantly decrease after addition of ciprofloxacin (0.1 μg/mL) or azithromycin (1.0 μg/mL).

3.5. Ciprofloxacin, azithromycin or doxycycline effect on *C. pneumoniae* induced IFNγ

When PBMC from asthmatic patients were infected with *C. pneumoniae*, levels of IFNγ produced were increased (8-fold) from baseline ($p = 0.03$) (Fig. 2). Levels of IFNγ decreased from these organism-infected levels in a non-significant manner after addition of 0.1 μg/mL ciprofloxacin or azithromycin, but significantly so with this dose of doxycycline ($p = 0.04$). Use of any of the drugs at 1.0 μg/mL imparted no significant reductions in these elevated values.

4. Discussion

The current study examined *in vitro* activities of ciprofloxacin, azithromycin, and doxycycline in *C. pneumoniae*-infected PBMC, as well as their ability to regulate cytokine (IFNγ and IL-4) and IgE responses. The results demonstrated that in this *ex-vivo* PBMC model: (1) only doxycycline (at 0.1 μg/mL) suppressed IFNγ production, (2) no drug had a significant effect on IL-4 production, and (3) none of the three drugs affected IgE production. The results here may assist in antibiotic selection for treatment of asthmatics with *C. pneumoniae* infection.

Prior literature has reported that treatment of *C. pneumoniae* and *M. pneumoniae* infections with antibiotics (quinolones, macrolides, tetracyclines) may have beneficial effects in asthmatics [2,7], as well as potential anti-inflammatory properties in addition to anti-chlamydial activity. However, this study is novel in that we looked at the *in vitro* effect of these 3 drug classes, which has not been evaluated in the context of cytokine or IgE responses. For this purpose, we established an experimental model based on our

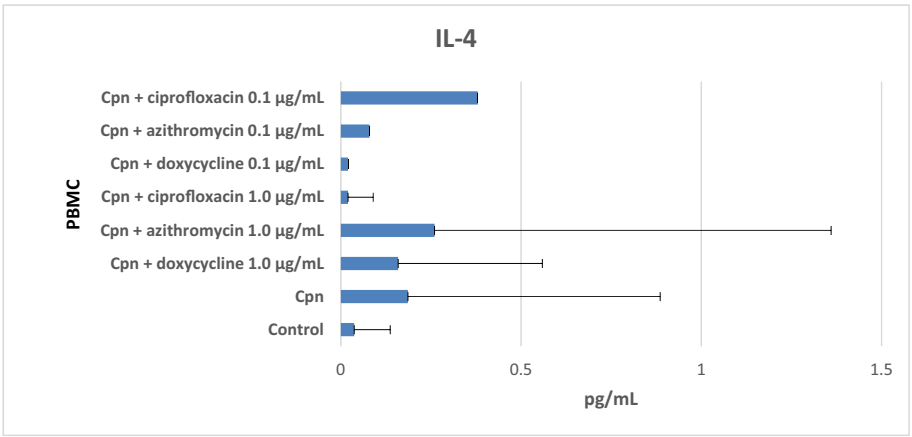

Fig. 1. Effect of ciprofloxacin, azithromycin or doxycycline on *C. pneumoniae* induced IL-4 responses. PBMC (1.5×10^6) from asthmatic patients (N = 18) were infected with *C. pneumoniae* (MOI = 0.1), and then cultured+/-ciprofloxacin (1.0 μg/mL), azithromycin (0.1 μg/mL), or doxycycline (0.1, 1.0 μg/mL). Levels of IL-4 were measured from supernatants collected on day 2 of culture (48 h) (ELISA). Data are expressed as pg/mL (mean + SD). Cpn: *C. pneumoniae*.

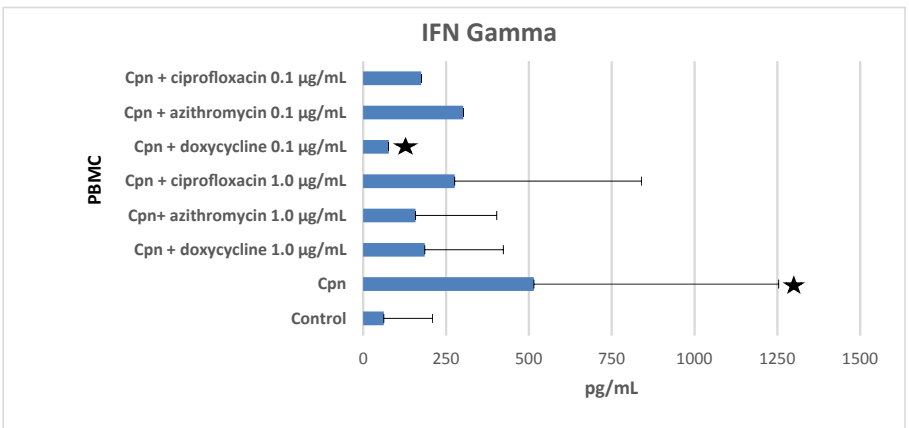

Fig. 2. Effect of ciprofloxacin, azithromycin or doxycycline on *C. pneumoniae* induced IFN-γ responses. PBMC (1.5×10^6) from asthmatic patients (N = 18) were infected with *C. pneumoniae* (MOI = 0.1), and then cultured+/-ciprofloxacin (1.0 μg/mL), azithromycin (0.1 μg/mL), or doxycycline (0.1, 1.0 μg/mL). Levels of IFN-γ were measured from supernatants collected on day 2 of culture (48 h) (ELISA). Data are expressed as pg/mL (mean + SD). Cpn: *C. pneumoniae*. Black star represents statistical significance P= <0.05.

Please cite this article in press as: Smith-Norowitz TA, et al., Doxycycline suppresses *Chlamydia pneumoniae* induced interferon-gamma responses in peripheral blood mononuclear cells in children with allergic asthma, J Infect Chemother (2018), https://doi.org/10.1016/j.jiac.2018.02.004

previous work [10,17], comprising treatment of *C. pneumoniae*-infected PBMC with ciprofloxacin, azithromycin or doxycycline, at two different concentrations.

It is well known that asthmatic patients are more susceptible to *C. pneumoniae* infections, and that infections are associated with a more severe, steroid resistant, non-eosinophilic phenotype of asthma [18–20]. Prior literature has reported an association between *C. pneumoniae* infection and exacerbations of asthma [1,2,20]. Antibiotic treatment of *C. pneumoniae* infection in asthmatics may lead to improvement of disease activity [20,21]. However, it should be mentioned, that macrolides, quinolones, and tetracyclines all have immunomodulatory activity which is independent of their antimicrobial activity [2,7]; positive outcomes from treatment may be due to immunomodulatory effects, anti-chlamydial effects or both [7].

Recent findings in our laboratory demonstrated *in vitro* suppression of *C. pneumoniae*-induced cytokine and IgE responses by doxycycline which was independent of anti-chlamydial activity in PBMC obtained from patients with asthma [10], suggesting that antibiotics have an important anti-inflammatory role in patients with asthma [10]. However, the observed differences from those studies might be due to other elements of inflammation in the group of patients, and further immunological studies are required to evaluate this issue.

Krakauer and Buckley demonstrated that doxycycline has anti-inflammatory effects *in vitro* (PBMC) and inhibits staphylococcal exotoxin-induced cytokines (IL-1β, IL-6, tumor necrosis factor (TNF)-α, IFN-γ) and chemokines (macrophage inflammatory protein (MIP)-1α, MIP-1β) [22]. Thus, the use of doxycycline can provide both anti-microbial and anti-inflammatory effects *in vitro*; however, anti-chlamydial effects were not studied.

Macrolides are effective against respiratory bacterial pathogens, including *C. pneumoniae* and *M. pneumoniae* [7,21]; these pathogens have been implicated in asthma exacerbation [3,6]. However, the efficacy of macrolides in clinical trials has been variable [7,21]. Other studies did not show a benefit of macrolide treatment in *C. pneumoniae* infected patients with asthma due to low numbers of study subjects with confirmed asthma [23,24]; a Cochrane database systemic review published in 2005 was inconclusive [9]. These discrepancies may be explained by the fact that asthma is a heterogeneous disease with different phenotypes and different responses to treatment [25]. It is well known that Th2 responses are important in the pathogenesis of allergic asthma [10], but few studies have examined the effects of macrolides on Th2 immune responses to *C. pneumoniae* infection in an *ex-vivo* PBMC model.

The most important finding in the current study was that doxycycline (0.1 μg/mL) suppressed IFN-γ production, while there was no significant effect on IL-4 production *in vitro*; IgE responses were not affected. Of notable interest, the standard deviation was much higher for both IL-4 and IFN-γ in the groups treated with higher doses of antibiotics. This may indicate greater variability in bacterial load in these groups or that these higher concentrations are having an effect on cell viability. However, it should be mentioned, that infection-induced IFN-γ can drive non-eosinophilic asthma responses [19,20]; therefore, antibiotic therapies may be useful in their treatment. Potential mechanisms for doxycycline suppression of *C. pneumoniae* induced- IFN-γ responses remain to be elucidated.

It could be that macrolides act on different pathways and thus affect different outcomes (Th1 and Th2 immunity) [26], or that macrolides produce different outcomes in stimulated compared with unstimulated cells [26]. We can also speculate that the cytokine responses observed in the current study likely reflect the cumulative responses of many cell types. Lin et al. demonstrated that azithromycin reduced Th0 and Th2 cell proliferation, T-cell viability, and increased apoptosis *ex vivo* in children with asthma [27], and that azithromycin decreased IL-5 production in Th2 cells, but did not affect IL-13 or IFN-γ responses [27]. Prior literature has focused on effect of macrolides on the innate immune system (macrolide-mediated modulation of monocytes) [28]. At the cytokine expression level, alterations of various pro-inflammatory cytokines, such as IL-1β, IL-8, IL-17 or TNF-α have been described [29]. In lung epithelial cells, azithromycin can inhibit activation of pro-inflammatory transcription factors (nuclear factor κβ (NF-κβ) and activator protein 1) [30]. The NF-κβ pathway is an important role in innate responses against respiratory viral infections and has been implicated in asthma pathogenesis [31].

Several important factors, including duration of infection before treatment and character of individual host responses, should be considered when assessing potential anti-inflammatory effects of drug outcome [32]. Through understanding a drug's effect on cytokine responses in patients, it should be considered that decreasing cytokine responses could have advantages and disadvantages to the host [32]. These cytokine responses might affect disease progression in a manner that may not be predicted by only its antimicrobial activity [32]. It remains to be determined whether the *in vitro* effect of azithromycin, ciprofloxacin, or doxycycline on *C. pneumoniae*-induced cytokine responses, results in beneficial effect for patients. However, it should be mentioned that ciprofloxacin does not effectively treat acute *C. pneumoniae* or *C. trachomatis* infections in patients [7,33,34]. *In vitro* studies have reported that ciprofloxacin treatment of *C. trachomatis* infected HEp-2 cells suppresses overt growth of the organism but does not completely eradicate viable bacterium [11]. Since clinical treatment is not effective [34,35] (based on MICs 2–4 μg/mL [7]), any observed responses in the present study after addition of ciprofloxacin, would most likely not be due to its anti-chlamydial activity. However, one could potentially use ciprofloxacin to augment conventional antibiotic treatment as an add-on or priming anti-inflammatory agent.

In the current study, *in vitro* activity of ciprofloxacin, azithromycin or doxycycline in *C. pneumoniae*-infected PBMC did not affect *in vitro* IgE responses. In our patient population, there were no patients that produced high IgE levels *in vitro*, after 10 days of stimulation. This finding is important because antibiotic therapies may prove to be more effective in the treatment of certain asthma phenotypes (non-eosinophilic/Th1/Th17 versus Th2). It is well known that cytokines participate in many physiologic and immunologic processes, including the regulation of inflammatory responses [35]; thus, the low levels of inflammatory responses we observed in infected and non-infected PBMC (before and after drug treatment) may be due to low levels of specific IgE, or unknown mechanisms responsible for regulation of pathogen clearance. Previous studies in our laboratory reported IgE production, along with a significant increase in IL-4 levels, and a correlation between levels of *C. pneumoniae*-induced IgE and IL-4, suggesting that *C. pneumoniae* infection mediates IgE production through Th2 lymphocyte activation [10], and that doxycycline suppresses *C. pneumoniae*-mediated increases in ongoing IgE and IL-4 responses by PBMC of patients with asthma [10]. This suppression was independent of doxycycline anti-chlamydial activity [10]. Earlier studies in our laboratory demonstrated that minocycline and doxycycline have the ability to suppress induction of IgE responses by PBMC obtained from asthmatic serum IgE + atopic donors (adult asthmatics) [36]. However, the differences observed may be due to selection of different populations (adult vs. pediatrics).

5. Conclusions

The findings presented in this study highlight for the first time, to our knowledge, the suppressive effect of doxycycline on

Please cite this article in press as: Smith-Norowitz TA, et al., Doxycycline suppresses *Chlamydia pneumoniae* induced interferon-gamma responses in peripheral blood mononuclear cells in children with allergic asthma, J Infect Chemother (2018), https://doi.org/10.1016/j.jiac.2018.02.004

C. pneumoniae induced IFN-γ responses in PBMC from children with allergic asthma. Drug trials evaluating antibiotics with potential anti-inflammatory effects in patients with asthma need to be based on pre-clinical studies identifying the optimal drug class and concentration for optimal impact, as potentially effective strategies for the treatment of asthma.

Ethics and consent to participate

The SUNY Downstate Medical Center Institutional Review Board (IRB) approved this study with the need for written informed consent to participate in the study.

Consent to publish

Not applicable. This manuscript does not contain any individual persons' data.

Competing interests

All authors declare no financial and non-financial competing interests.

Funding source

This work was supported, in part by the Thrasher Research Fund (Young Investigator Award to Stephan Kohlhoff, M.D.).

Transparency declaration

None to declare.

Acknowledgements

We would like to thank Kevin Norowitz, M.D. (SUNY Downstate Med Center, Dept. of Pediatrics) for fruitful discussions.

References

[1] Hammerschlag MR, Kohlhoff SA, Gaydos CA. Chlamydia pneumoniae. In: Mandell GL, Bennett JE, Dolin R, editors. Principles and practice of infectious diseases. eighth ed. Philadelphia, PA: Elsevier, Inc.; 2014. p. 2174–82.

[2] Johnston SL, Martin RJ. *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* a role in asthma pathogenesis? Am J Respir Crit Care Med 2005;172:1078–89.

[3] Emre U, Roblin PM, Gelling M, Dumornay W, Rao M, Hammerschlag MR, et al. The association of *Chlamydia pneumoniae* infection and reactive airway disease in children. Arch Pediatr Adolesc Med 1994;148:727–31.

[4] Hammerschlag MR, Chirgwin K, Roblin PM, Gelling M, Dumornay W, Mandel L, et al. Persistent infection with *Chlamydia pneumoniae* following acute respiratory illness. Clin Infect Dis 1992;14:178–82.

[5] Martin RJ, Kraft M, Chu HW, Berns EA, Cassell GH. A link between chronic asthma and chronic infection. J Allergy Clin Immunol 2001;107:595–601.

[6] Ikezawa S. Prevalence of Chlamydia pneumoniae in acute respiratory tract infection and detection of anti-*Chlamydia pneumoniae*-specific IgE in Japanese children with reactive airway disease. Kurume Med J 2001;48(2):165–70.

[7] Kohlhoff SA, Hammerschlag MR. Treatment of chlamydial infections: 2014 update. Expert Opin Pharmacother 2015;16:205–12.

[8] Daoud A, Gloria CJ, Taningco G, Hammerschlag MR, Weiss S, Gelling M, et al. Minocycline treatment results in reduced oral steroid requirements in adults. Asthma Allergy Asthma Proc 2008;29:286–94.

[9] Richeldi L, Ferrara G, Fabbri LM, Lasserson TJ, Gibson PG. Macrolides for chronic asthma. Cochrane Database Syst Rev 2005;(issue 4):CD002997.

[10] Dzhindzhikhashvili MS, Joks R, Smith-Norowitz TA, Durkin HG, Chotikanatis K, Estrella E, et al. Doxycycline suppresses *Chlamydia pneumoniae*-mediated increases in ongoing immunoglobulin E and interleukin-4 responses by peripheral blood mononuclear cells of patients with allergic asthma. J Antimicrob Chemother 2013;68:2363–8.

[11] Dreses-Werringloer U, Padubrin I, Jürgens-Saathoff B, Hudson AP, Zeidler H, Köhler L. Persistence of *Chlamydia trachomatis* is induced by ciprofloxacin and ofloxacin in vitro. Antimicrob Agents Chemother 2000;44:3288–97.

[12] Cowen MK, Wakefield DB, Cloutier MM. Classifying asthma severity: objective versus subjective measures. J Asthma 2007;44:711–5.

[13] Wang SP, Grayston JT. Microimmunofluorescence serological studies with the TWAR organism. In: Oriel D, Ridgeway G, editors. Chlamydial infections: Proceedings of the Sixth International Symposium on human chlamydial infections. Cambridge: Cambridge University Press; 1986. p. 329–32.

[14] Roblin PM, Dumornay W, Hammerschlag MR. Use of HEp-2 cells for improved isolation and passage of *Chlamydia pneumoniae*. J Clin Microbiol 1992;1992(30):1968–71.

[15] Apfalter P, Barousch W, Nehr M, Makristathis A, Willinger B, Rotter M, et al. Comparison of a new quantitative *ompA*-based real-time PCR TaqMan assay for detection of *Chlamydia pneumoniae* DNA in respiratory specimens with four conventional PCR assays. J Clin Microbiol 2003;41:592–600.

[16] Waring AL, Halse TA, Csiza CK, Carlyn CJ, Arruda Musser K, Limberger RJ. Development of a genomics-based PCR assay for detection of *Mycoplasma pneumoniae* in a large outbreak in New York State. J Clin Microbiol 2001;39:1385–90.

[17] Kutlin A, Roblin PM, Hammerschlag MR. In vitro activity of azithromycin and ofloxacin against *Chlamydia pneumoniae* in a continuous infection model. Antimicrob Agents Chemother 1999;43:2268–72.

[18] Sandoz KM, Rockey DD. Antibiotic resistance in Chlamydiae. Future Microbiol 2010;5:1427–42.

[19] Robinson D, Humbert M, Buhl R. Revisiting type 2-high and type 2-low airway inflammation in asthma: current knowledge and therapeutic implications. Clin Exp Allergy 2017;47:161–75.

[20] Hammerschlag MR, Kohlhoff SA. Treatment of chlamydial infections. Expert Opin Pharmacother 2011;13:542–52.

[21] Hammerschlag MR, Kohlhoff SA, Darville T. Chlamydia pneumoniae and Chlamydia trachomatis. In: Fratamico PM, Smith JL, Brogden KA, editors. Sequelae and long-term consequences of infectious diseases. Washington, DC: American Society for Microbiology; 2009. p. 27–52.

[22] Krakauer T, Buckley M. Doxycycline is anti-inflammatory and inhibits staphylococcal exotoxin-induced cytokines and chemokines. Antimicrob Agents Chemother 2003;47(11):3630–3.

[23] Johnston SL, Blasi F, Black PN, Martin RJ, Farrell DJ, Nieman RB, et al. The effect of telithromycin in acute exacerbations of asthma. N Engl J Med 2006;354:1589–600.

[24] Sutherland ER, King TS, Icitovic N, Ameredes BT, Bleecker E, Boushey HA, et al. A trial of clarithromycin for the treatment of suboptimally controlled asthma. J Allergy Clin Immunol 2010;126:747–53.

[25] Wenzel SE. Complex phenotypes in asthma: current definitions. Pulm Pharmacol Ther 2013;26:710–5.

[26] Wong EHC, Porter JD, Edwards MR, Johnston SL. The role of macrolides in asthma: current and evidence and future directions. Lancet Respir Med 2014;2:657–70.

[27] Lin SJ, Lee WJ, Liang YW, Yan DC, Cheng PJ, Kuo ML. Azithromycin inhibits IL-5 production of T helper type 2 cells from asthmatic children. Int Arch Allergy Immunol 2011;156:179–86.

[28] Khan AA, Slifer TR, Araujo FG, Remington JS. Effect of clarithromycin and azithromycin on production of cytokines by human monocytes. Int J Antimicrob Agents 1999;11:121–32.

[29] Vanaudenaerde BM, Wuyts WA, Geudens N. Macrolides inhibit IL17-induced IL8 and 8-isoprostane release from human airway smooth muscle cells. Am J Transplant 2007;7:76–82.

[30] Zhu C, Lei W, Huang J. Azithromycin inhibits double-stranded RNA-induced thymic stromal lymphopoietin release from human airway epithelial cells. Pharmazie 2013;68. 899–03.

[31] Tully JE, Hoffman SM, Lahue KG, Nolin JD, Anathy V, Lundblad LK, et al. Epithelial NF-kappa B orchestrates house dust mite-induced airway inflammation, hyperresponsiveness, and fibrotic remodeling. J Immunol 2013;191:5811–21.

[32] Purswani M, Eckert S, Arora H, Johann-Liang R, Noel GJ. The effect of three broad-spectrum antimicrobials on mononuclear cell responses to encapsulated bacteria: evidence for down-regulation of cytokine mRNA transcription by trovafloxacin. J Antimicrob Chemother 2000;46:921–9.

[33] Skerk V, Schonwald S, Krhen I, Banaszak A, Begovac J, Strugar J, et al. Comparative analysis of azithromycin and ciprofloxacin in the treatment of chronic prostatitis caused by Chlamydia trachomatis. Int J Antimicrob Agents 2003;21:457–62.

[34] Hooton TM, Rogers ME, Medina TG, Kuwamura LE, Ewers C, Roberts PL, et al. Ciprofloxacin compared with doxycycline for nongonococcal urethritis. Ineffectiveness against *Chlamydia trachomatis* due to relapsing infection. J Am Med Assoc 1990;264:1418–21.

[35] Geng Y, Berencsi K, Gyulai Z, Gonczol E, Zaki MH, Margolis DJ, et al. Roles of interleukin-12 and gamma interferon in murine *Chlamydia pneumoniae* infection. Infect Immun 2000;68:2245–53.

[36] Smith-Norowitz TA, Bluth MH, Drew H, Norowitz KB, Chice S, Shah VN, et al. Effect of minocycline and doxycycline on IgE responses. Ann Allergy Asthma Immunol 2002;89:172–9.

Please cite this article in press as: Smith-Norowitz TA, et al., Doxycycline suppresses *Chlamydia pneumoniae* induced interferon-gamma responses in peripheral blood mononuclear cells in children with allergic asthma, J Infect Chemother (2018), https://doi.org/10.1016/j.jiac.2018.02.004